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Development of MAPC derived induced endodermal progenitors

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Chapter 5

Epigenetic induction of definitive and pancreatic endoderm cell fate in human fibroblasts

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Chapter 5

Epigenetic induction of definitive and pancreatic endoderm cell fate in human fibroblasts

5.1 Abstract

Reprogramming can occur by the introduction of key transcription factors (TFs) as well as by epigenetic changes. We demonstrated that histone deacetylase inhibitor (HDACi) Trichostatin A (TSA) combined with a chromatin-remodeling medium (CRM) induced expression of a number of definitive endoderm and early and late pancreatic marker genes. When CRM was omitted, endoderm/pancreatic marker genes were not induced. Furthermore, treatment with DNA methyltransferase inhibitor (DNMTi) 5-azacytidine (5AZA) CRM did not affect gene expression changes, and when 5AZA was combined with TSA, no further increase in gene expression of endoderm, pancreatic endoderm, and endocrine markers was seen over levels induced with TSA alone. Interestingly, TSA-CRM did not affect expression of pluripotency and hepatocyte genes but induced some mesoderm transcripts. Upon removal of TSA-CRM, the endoderm/pancreatic gene expression profile returned to baseline. Our findings underscore the role epigenetic modification in transdifferentiation of one somatic cell into another. However, full reprogramming of fibroblasts to β -cells will require combination of this approach with TF overexpression and/or culture of the partially reprogrammed cells under β -cell specific conditions.

5.2 Introduction

Type-1 Diabetes mellitus (DM-I) is a severe metabolic disease that affects millions of people worldwide. It involves complete loss of functional insulin secreting β -cells in the pancreas due to autoimmune destruction. DM-1 leads to hyperglycemia, which can be treated with insulin injections. However in the long-term, DM-1 leads to micro and macro vascular, cardiovascular, neuronal, renal and ocular complications due to intermittent hyperglycemia. Replacement of the destroyed β -cells is the only

curative treatment. However, limited numbers of available donor organs and immunological issues restrict whole pancreas or islet transplantation [1, 2]. An alternative source for human cadaveric islets is generating insulin-producing β -cells from stem cells and/or somatic cells. However, the challenge in this area is to identify an adequate stem or progenitor cells and mechanisms to create a safe source of mature insulin-producing β -cells from such cells.

Over the last decade several studies have demonstrated that it is possible to generate functional β -cells from human embryonic stem cells (hESCs) by culturing the cells in conditions that mimic *in vivo* pancreatic development [3-6]. With the advent of induced Pluripotent Stem Cells (iPSCs) technology, developed by the Yamanaka team [7, 8], it has now also become possible to create Human Leukocyte Antigen (HLA)-identical β -cells to treat DM-I. However, the pluripotent nature of ESCs and iPSCs leaves the possibility for teratoma formation [6, 9] if full differentiation towards β -cells is not achieved. An alternative approach is to transdifferentiate somatic cells into insulin producing β -cells without passing through a pluripotent state, via ectopic expression of defined TFs and culture in supportive medium. A number of studies demonstrated in rodents that introduction of a single (*PDX1*) or group of TFs (*PDX1*, *NGN3*, *MAFA*) can transdifferentiate hepatocytes, intestinal cells, exocrine acinar or ductal pancreatic cells, or endocrine α -cells, thereby reprogramming these non- β -cell endodermal cells into β -cells [10-17]. In addition, Li *et al.*, created endodermal progenitor cells by transiently overexpressing *OCT4*, *SOX2*, *KLF4* and *CMYC* (OKSM) in mouse embryonic fibroblasts (MEFs) combined with small molecule epigenetic modifiers, which could subsequently be converted to β -cells [18]. Several recent studies have treated human or swine fibroblasts [19-21], mesenchymal stem cells [22], or rat liver stem cells [23], with epigenetic modifying molecules (DNA methyltransferase inhibitor and/or histone deacetylase inhibitor in CRM followed by culture under β -cell specifying conditions. This combined treatment resulted in the generation of endocrine pancreatic β -cells that reversed hyperglycemia in immunodeficient mice. Moreover, epigenetic modification also induced *NGN3* expression and endocrine differentiation of the PNAC-1 human ductal cell line [24]. However many studies

reported that TSA itself could induce chromatin changes without the presence of chromatin remodeling medium (CRM). For instance, using fluorescence anisotropy imaging and fluorescence recovery after photo-bleaching (FRAP) and fluorescence correlation spectroscopy (FCS), it was demonstrated that TSA induced histone protein dynamics and expression in HeLa cells by increasing the euchromatin fraction and increasing core acetylation patterns, phosphorylation patterns, and nuclear volume [25]. Similarly TSA induces histone acetylation and reversible decondensation of interphase chromatin structure in HeLa cells, as demonstrated by image correlation spectroscopy (ICS) and spatially resolved scaling analysis (SRSA) methods [26]. In another study human hepatocellular carcinoma (HepG2) and NIH 3T3 cells, treatment with TSA and VPA (Valproic acid) increased the active chromatin marks, such as H3K9ac and H3K4me2 abundance, which might lead to chromatin decondensation [27, 28].

We here optimized the exposure of human fibroblasts to epigenetic modifiers to convert them to endoderm and pancreatic endocrine progenitors. We demonstrated that culture of adult human and foreskin fibroblasts with TSA combined with a chromatin remodeling medium (CRM), induces expression of endoderm and pancreatic endoderm genes, but that this is a transient phenomenon. Hence, further maintenance in β -cell-specifying conditions with or without forced expression of exogenous TFs will be needed to permanently convert fibroblasts to β -cells.

5.3 Materials and methods

5.3.1 Culture of BJ1 human fibroblasts

Human BJ1 adult fibroblasts were cultured in 90% DMEM-F12 + HEPES (Cat. No. 11330032, Gibco, Grand Island, NY - USA), 10% Fetal Bovine Serum (FBS)(Cat.No. F6178, Sigma-Aldrich, Saint Louis, MO, USA) and 1% Penicillin/Streptomycin (Cat.No. 15140122, Gibco, Carlsbad, CA, USA). Cells were maintained under normoxia conditions, 37°C temperature and 5% CO₂.

5.3.2 Isolation and culture of primary human fibroblasts

A skin biopsy from healthy volunteer/ non-diabetic donor was obtained following informed consent and with approval from the ethical committee of University of KU Leuven. Fibroblasts were isolated in minimum DMEM (Cat.No.31885049, Gibco, Grand Island, NY, USA) supplemented with 20% FBS. After four passages, fibroblasts were frozen in liquid nitrogen in several aliquots. After thawing cells were grown in 90% DDM HG + Glutamax™, pyruvate (Cat.No. 31966047, Gibco, Grand Island, NY - USA), supplemented with 10% FBS (Cat.No. F6178, Sigma-Aldrich, Saint Louis, MO, USA), and 1% Penicillin/ Streptomycin (Cat.No. 15140122, Gibco, Carlsbad, CA, USA). Cells were maintained under normoxia conditions, 37°C temperature and 5% CO₂.

5.3.3 Reprogramming conditions

Chromatin remodeling medium (CRM) consisted of 10% knockout™ (KO) serum (Cat.No. 10828028, Gibco, Grand Island, NY – USA), Knockout™- DMEM (Cat.No. 10829018, Gibco, Grand Island, NY – USA), 50 µM, β-mercaptoethanol (50mM) (Cat.No. 31350010, Gibco, Grand Island, NY, USA), 1% MEM-Non-Essential Amino Acids solution (100x) (Cat.No. 11140050, Gibco, Grand Island, NY – USA), 1% B27® supplement (Cat.No. 17504044, Gibco, Grand Island, NY – USA), 2 mM L-glutamine (200mM) (Cat.No. 25030081, Paisley, Scotland, UK), 2% N2-supplement (100x)(Cat.No. 17502001, Gibco, Grand Island, NY – USA) all from Gibco, Life Technologies(Thermo Scientific), and 20 ng/ml recombinant human basic fibroblast growth factor (Cat.No. 100-18C, Peprotech, USA), 20 ng/ml epidermal growth factor (Cat.No.236-EG, R&D Systems, MN-USA), 1000 U/ml penicillin and Streptomycin (10,000U/ml) (Cat.No. 15140122, Gibco, Carlsbad, CA, USA), and 100nM L-Ascorbic Acid (Cat.No. A4403, Sigma-aldrich, Saint Louis, MO, USA). Cells were plated in 6 well

plate (Cat.No. 3516, Corning-Costar®, MA, USA) and exposed to 3 μ M or 5 μ M 5-AZA (Cat.No. A2385, Sigma Aldrich, Saint Louis, MO, USA) dissolved in DMSO (Cat.No.D2650, Sigma-Aldrich, Saint Louis, MO, USA) in CRM medium for 48 hours, with medium change at 24h. For treatment with Trichostatin A (TSA) - ready made solution 5mM (Cat.No. T1952, Sigma Aldrich, Saint Louis, MO, USA), cells were exposed to various concentrations between 100 nM to 100 μ M for 24 hours. Untreated fibroblasts and DMSO treated fibroblasts cultured in CRM were used as controls.

5.3.4 RNA isolation, cDNA synthesis and quantitative real time- polymerase chain reaction (qRT-PCR)

Total RNA was extracted using GenElute™ Mammalian Total RNA Miniprep Kit (Cat.No. RTN350, Sigma-Aldrich, Saint Louis, MO, USA), for sample size $<10^5$ cells, ZR RNA Microprep™ CA-USA (Cat.No. R1061, Zymo Research, CA, USA), was used and cDNA was synthesized from 500ng-1 μ g total RNA using SuperScript® III First-Strand Synthesis SuperMix for qRT-PCR kit (Cat.No. 11752050, Invitrogen, CA, USA), according to manufacturer's protocol. For qPCR: the cDNA underwent 40 rounds of amplification cycles on a ViiA™ 7 Real-Time PCR System with 384-well plate (Cat.No. 4453536, Applied Biosystems, Carlsbad, CA, USA) as follows: 40 cycles of a 2-step PCR (95°C for 15 sec; 60 °C for 45 sec) after initial denaturation (50 °C for 2 min, 95°C for 2 min) using specific primers, Platinum® SYBR® Green qPCR SuperMix-UDG w/ROX (Cat.No. 11744500, Invitrogen, CA, USA) and 2 μ l cDNA. For normalization purposes, *GAPDH* (Glyceraldehyde 3-phosphate dehydrogenase) were used as a housekeeping control and results are shown in relative expression to *GAPDH*. All Primers were synthesized at (IDT technologies, Leuven, Belgium). The cycle threshold value >40 are considered undetectable and calculated as Ct of 40. A list of the primers can be found below (Supplementary table-1).

5.3.5 Statistical analysis: Parametric distribution of data points was confirmed using the Kolmogorov-Smirnov test. Comparisons between two groups were analyzed using an unpaired 2-tailed Student's t-test. P-values < 0.05(*), < 0.01(**), <0.001(***) were considered significant. Data are shown as mean and error bars represent standard error of mean (SEM) of minimum three independent experiments. All results were analyzed using Graph Pad prism 6 software.

5.4 Results

5.4.1 Treatment of human primary adult and foreskin BJ fibroblasts with TSA induced definitive endoderm and pancreatic endocrine genes, but only transiently

We initially tested the effect of addition of the HDAC inhibitor, TSA for 24h, on human primary adult and BJ foreskin fibroblasts, cultured in chromatin remodeling medium (CRM). We then assessed levels of transcripts of definitive endoderm, early and late pancreatic endocrine markers, as well as hepatocyte, skeletal muscle, endothelium, and pluripotency marker genes. TSA/CRM significantly induced the expression of the early endoderm marker genes, *GATA4*, *EOMES*, *E-CADHERIN*, *SOX17*, *FOXA2*, and *CXCR4*, in both cell populations while induction of *SOX7* and *MIXL1* was only seen in primary human fibroblasts (Fig. 1A, Fig. 2A). In addition, expression of the early and late pancreatic progenitor and endocrine marker genes, *PTF1A*, *HLXB9*, *NKX6.1*, *ISL1*, *ARX*, and *MAFB* was observed in both cell populations, while *PDX1* expression was only detected in primary human fibroblasts treated cells (Fig. 1B-C, Fig. 2B-C). Transcripts for mature endocrine pancreatic cells including *PAX4*, *NGN3*, *INS*, *GCG*, and *SST* were however not increased following TSA/CRM culture (Fig. 1C, Fig. 2C). Transcripts for the hepatocyte marker genes, *ALB*, *AFP*, and *HNF4A* (Fig. 1D, Fig. 2D) were not expressed. We also assessed the effect of TSA/CRM on expression of mesodermal lineage transcripts and found an increase in *MYOD1* and *FLK1* expression but not the endothelium marker genes *TIE2*, *VE-CADHERIN* (Fig. 1E, Fig. 2E). Pluripotency marker genes *OCT4*, *SOX2*, and *NANOG* (Fig. 1F, Fig. 2F) were not induced. When the remodeling medium was removed, expression of the pancreatic endodermal genes was not maintained.

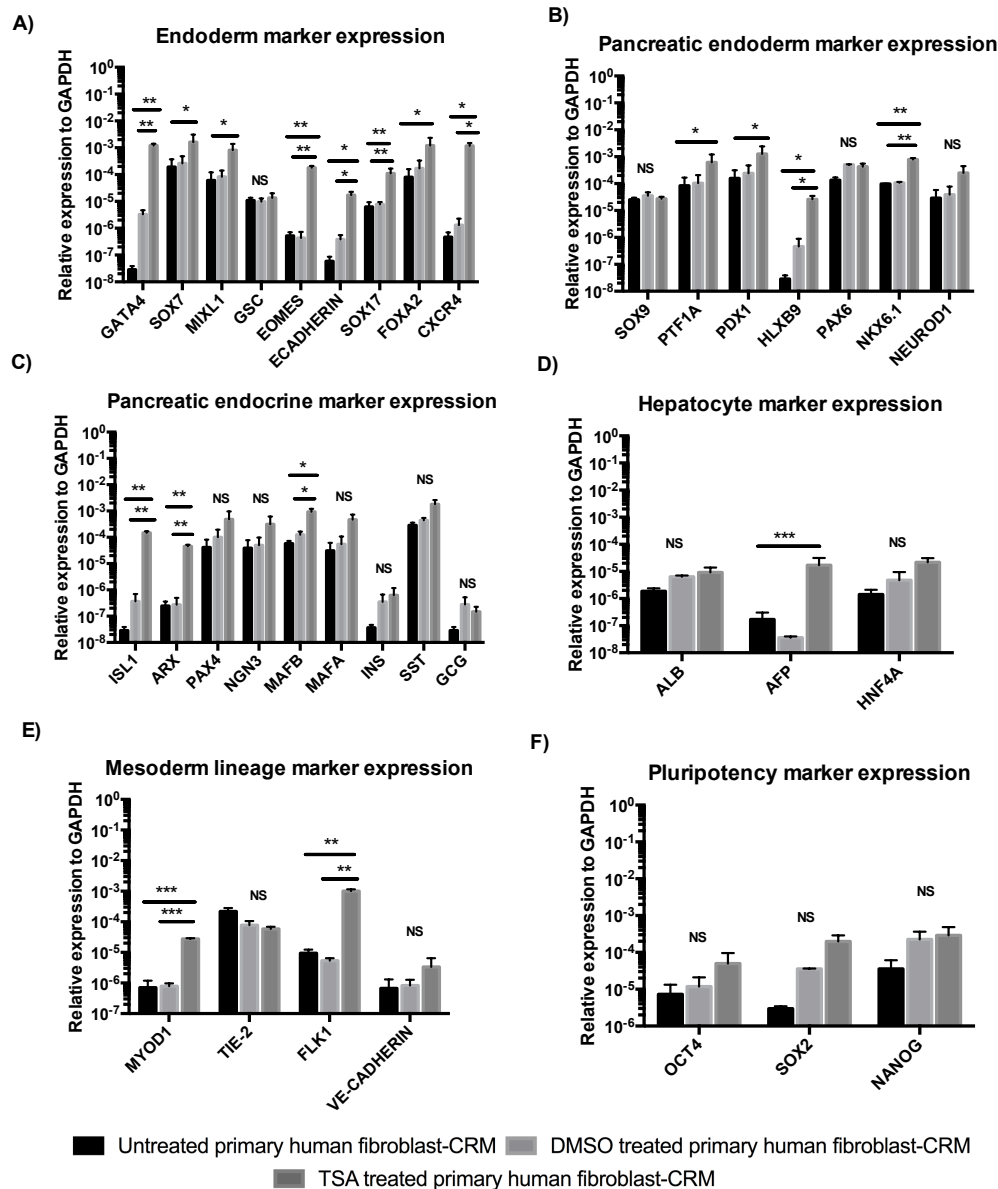


Figure-1: Trichostatin A (TSA) treatment of primary adult fibroblasts induces transient definitive endoderm and pancreatic endoderm markers. A) qRT-PCR analysis demonstrated induction of endodermal genes (*GATA4*, *SOX7*, *MIXL1*, *EOMES*, *E-CADHERIN*, *SOX17*, *FOXA2*, *CXCR4*, but not *GSC*) in TSA-CRM treated primary human fibroblast. B) qRT-PCR analysis demonstrated induction of pancreatic endoderm genes (*PTF1A*, *PDX1*, *HLXB9*, *NKX6.1*, but not *SOX9*, *PAX6*, *NEUROD1*) in TSA-CRM treated primary human fibroblast. C) qRT-PCR analysis demonstrated induction of pancreatic endocrine genes (*ISL1*, *ARX*, *MAFB*, but not *PAX4*, *NGN3*, *MAFA*, *INS*, *SST*, *GCG*) in TSA-CRM treated primary human fibroblast. D) qRT-PCR analysis demonstrated induction of hepatocyte genes (*ALB* and *HNF4A*) were not induced except for *AFP*. E) qRT-PCR analysis demonstrated induction of mesoderm lineage genes (*MYOD1* and *FLK1* but not *TIE-2* and *VE-CADHERIN*) in TSA-CRM treated primary human fibroblast. F) qRT-PCR analysis demonstrated no induction of pluripotency genes (*OCT4*, *SOX2*, *NANOG*) expression in TSA-CRM treated primary human fibroblast. Black bar – CRM treated primary human fibroblast cells; Light grey bar-DMSO-CRM primary human fibroblast treated cells; Dark grey bar-100μM TSA-CRM treated primary human fibroblast cells. Gene expression is shown relative to the housekeeping gene *GAPDH*. Data represent the mean \pm SEM (standard error of mean) of three independent experiments. Statistical significance tests were performed between TSA treated versus untreated fibroblast, and TSA treated versus DMSO treated fibroblast. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ by unpaired 2-tailed Student's t-test. NS- not significant.

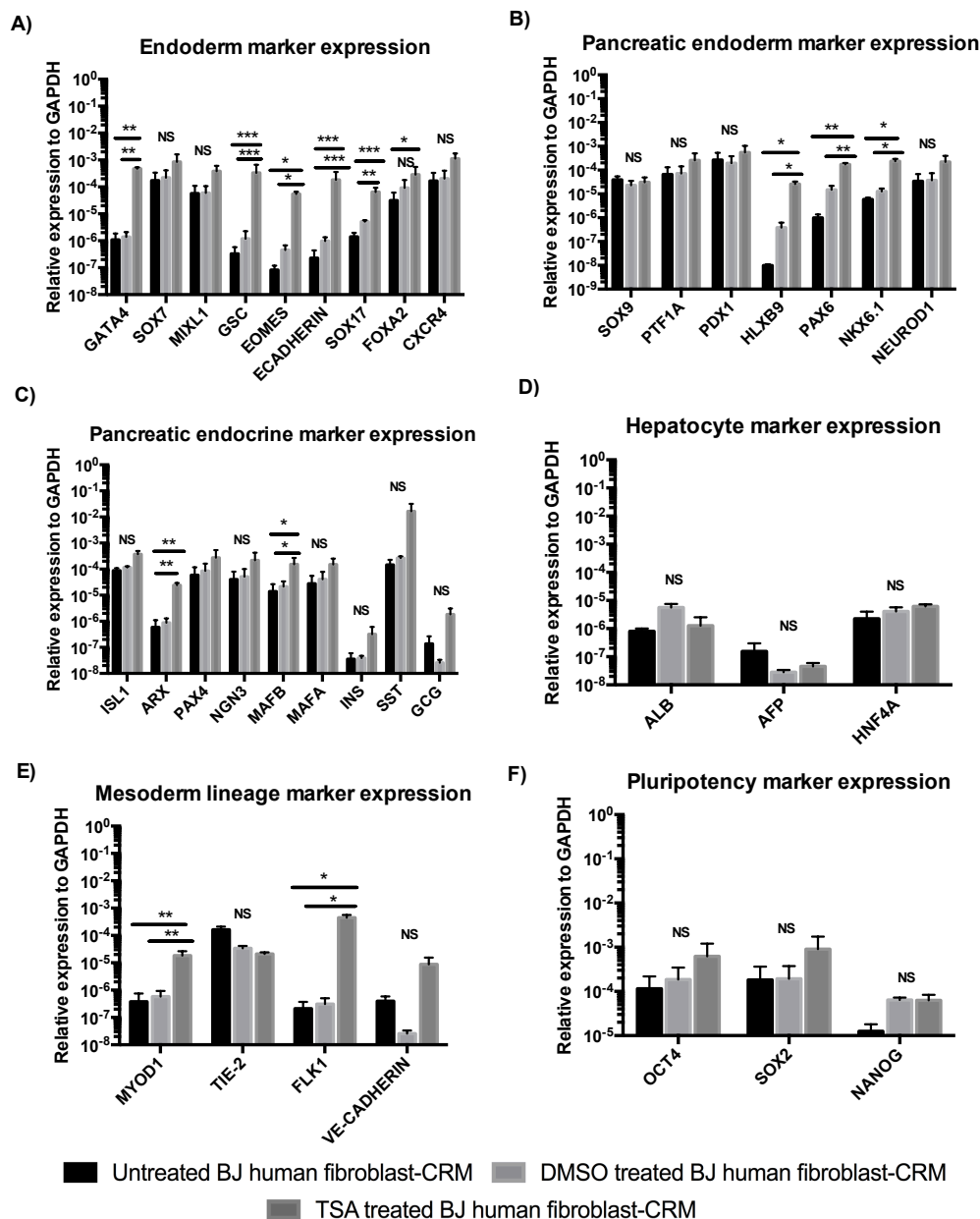


Figure-2: Trichostatin-A (TSA) treatment of BJ foreskin fibroblast induces transient definitive endoderm and pancreatic endoderm markers. A) qRT-PCR analysis demonstrated induction of endodermal genes (*GATA4*, *GSC*, *EOMES*, *E-CADHERIN*, *SOX17*, *FOXA2*, but not *SOX7*, *MIXL1*, *CXCR4*) in TSA-CRM treated BJ foreskin fibroblast. B) qRT-PCR analysis demonstrated induction of pancreatic endoderm genes (*HLXB9*, *PAX6*, *NKX6.1*, but not *SOX9*, *PTF1A*, *PDX1*, *NEUROD1*) in TSA-CRM treated BJ foreskin fibroblast. C) qRT-PCR analysis demonstrated induction of pancreatic endocrine genes *ARX*, *MAFB*, but not *ISL1*, *PAX4*, *NGN3*, *MAFA*, *INS*, *SST*, *GCG*) in TSA-CRM treated BJ foreskin fibroblast. D) qRT-PCR analysis demonstrated no induction of hepatocyte marker genes (*ALB*, *AFP*, *HNF4A*) in TSA-CRM treated BJ foreskin fibroblast. E) qRT-PCR analysis demonstrated induction of mesoderm lineage genes (*MYOD1* and *FLK1* but not *TIE-2* and *VE-CADHERIN*) in TSA-CRM treated BJ foreskin fibroblast. F) qRT-PCR analysis demonstrated no induction of pluripotency genes (*OCT4*, *SOX2*, *NANOG*) in TSA-CRM treated BJ foreskin fibroblast. Black bar – CRM treated BJ foreskin fibroblast cells; Light grey bar-DMSO-CRM treated BJ foreskin fibroblast cells; Dark grey bar-100μM TSA-CRM treated BJ foreskin fibroblast cells. Gene expression is shown relative to the housekeeping gene *GAPDH*. Data represent the mean ± SEM (standard error of mean) of three independent experiments. Statistical significance tests were performed in between TSA treated versus untreated and TSA treated versus DMSO treated. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ by unpaired 2-tailed Student's t-test. NS- not significant.

5.4.2 Combined treatment of fibroblasts with 5AZA and TSA did not result in further increased expression of definitive endoderm and pancreatic endocrine genes.

To test if combination of DNA methylation modification and inhibition of HDAC would induce a significant transdifferentiation of fibroblasts to endocrine pancreas, we next cultured primary adult fibroblasts and BJ fibroblast cells in CRM supplemented for 2 days with 3 or 5 μ M 5AZA for 48 hours, followed by TSA for 24 hours. However, as significant cell death was seen with 5AZA at 5 μ M or more, studies were done using 3 μ M 5AZA. Treatment for 2 days with 5AZA induced subtle morphological changes. In comparison with the typical elongated morphology of untreated fibroblasts 5AZA treated cells had a more rounded shape without long processes. 5AZA also decreased cell proliferation, which was analyzed by cell counting (data not shown). Following treatment with TSA on day 3, the cells appeared more flattened with granular cytoplasm and larger nuclei possibly due to more relaxed chromatin structure. Cell proliferation remained stable upon exposure to TSA.

We next assessed the effect of 5AZA combined with TSA on transcript expression as described for TSA only studies above. By contrast, following treatment with 5AZA followed by TSA, endodermal early and late pancreatic endocrine progenitor marker genes were induced in both primary and BJ foreskin fibroblasts (Fig. 3A-C, Fig. 4A-C). However, as with TSA only in this case also, mature pancreatic endocrine marker genes were not induced (Fig. 3C, Fig. 4C). As was seen for cultures treated with TSA alone, hepatocyte marker genes were not induced (Fig. 3D, Fig. 4D) but the mesodermal markers, *MYOD1* and *FLK1* were induced (Fig. 3E, Fig. 4E). Other mesodermal (Fig. 3E, Fig. 4E) and pluripotency marker genes (Fig. 3F, Fig. 4F) were also not induced. Again, when the epigenetic modifiers were removed, expression of the pancreatic endodermal genes was not maintained. Finally, we also demonstrated that aside from TSA, CRM was essential for induction of endodermal and pancreatic endodermal transcripts and that induction of the pancreatic endoderm genes was more efficient when early passage cells were used. We next assessed if combining 5AZA with TSA-CRM increases the expression level of definitive endoderm, early and later pancreatic endocrine progenitor transcripts. In both cell lines induction of

most, albeit not all, endodermal and early and late pancreatic marker genes were not improved by pretreatment of the cells with 5AZA before addition of TSA-CRM (Fig. 5-6A-C). 5AZA pretreatment enhanced expression of the skeletal muscle marker *MYOD1* (Fig. 5E), but not hepatocyte and pluripotency marker genes (Fig. 5-6D and F). Despite the morphological changes observed following treatment with 5AZA-CRM for 2 days, no induction of pancreatic or other lineage specific markers was observed (Fig. 7-8).

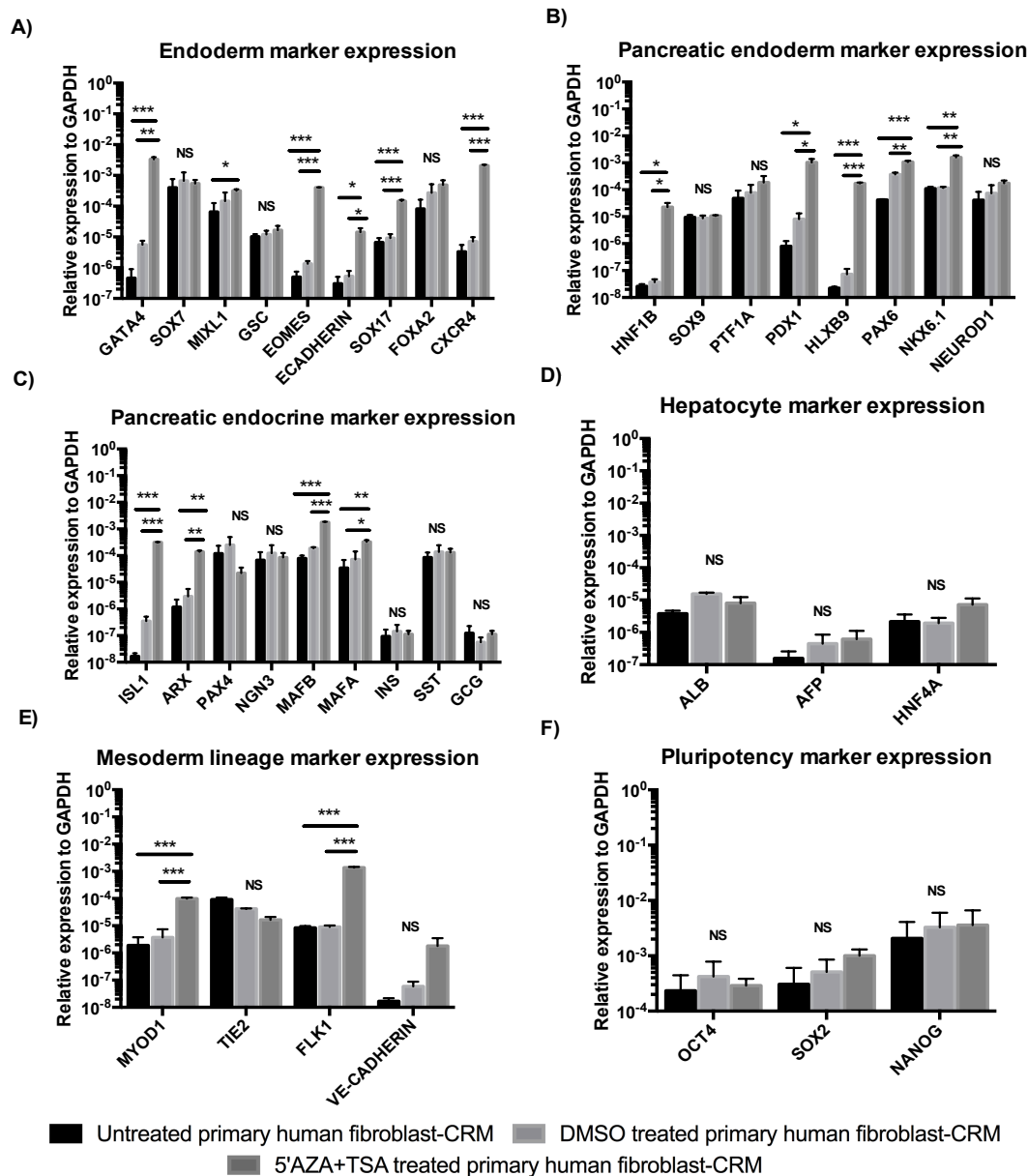


Figure-3: 5-azacytidine (5AZA) and Trichostatin A (TSA) treatment of primary adult fibroblast induces transient definitive endoderm and pancreatic endoderm markers. A) qRT-PCR analysis demonstrated induction of endodermal genes (*GATA4*, *MIXL1*, *EOMES*, *ECADHERIN*, *SOX17*, *CXCR4*, but not *SOX7*, *GSC*, *FOXA2*) in 5AZA+TSA CRM treated primary human fibroblast cells. B) qRT-PCR analysis demonstrated induction of pancreatic endoderm genes (*HNF1B*, *PDX1*, *HLXB9*, *PAX6*, *NKX6.1*, but not *SOX9*, *PTF1A*, *NEUROD1*) in 5AZA+ TSA CRM treated primary human fibroblast cells. C) qRT-PCR analysis demonstrated induction of the pancreatic endocrine genes (*ISL1*, *ARX*, *MAFB*, *MAFA* but not *PAX4*, *NGN3*, *INS*, *SST*, *GCG*) in 5AZA+ TSA CRM treated primary human fibroblast cells. D) qRT-PCR analysis demonstrated no induction of hepatocyte genes (*ALB*, *AFP*, *HNF4A*) in 5AZA+ TSA CRM treated primary human fibroblast cells. E) qRT-PCR analysis demonstrated induction of mesoderm lineage genes (*MYOD1* and *FLK1* but not *TIE-2* and *VE-CADHERIN*) in 5AZA+ TSA CRM treated primary human fibroblast cells. F) qRT-PCR analysis demonstrated no induction of pluripotency genes (*OCT4*, *SOX2*, *NANOG*) in 5AZA+ TSA CRM treated primary human fibroblast cells. Black bar – CRM treated primary human fibroblast cells; Light grey bar- DMSO-CRM treated primary human fibroblast cells; Dark grey bar- 3 μ M 5AZA-CRM followed by 100 μ M TSA CRM treated primary human fibroblast cells. Gene expression is shown relative to the housekeeping gene *GAPDH*. Data represent the mean \pm SEM (standard error of mean) of three independent experiments. Statistical significance tests were performed in between 5AZA+TSA treated versus untreated and 5AZA+TSA treated versus DMSO treated. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ by unpaired 2-tailed Student's t-test. NS- not significant.

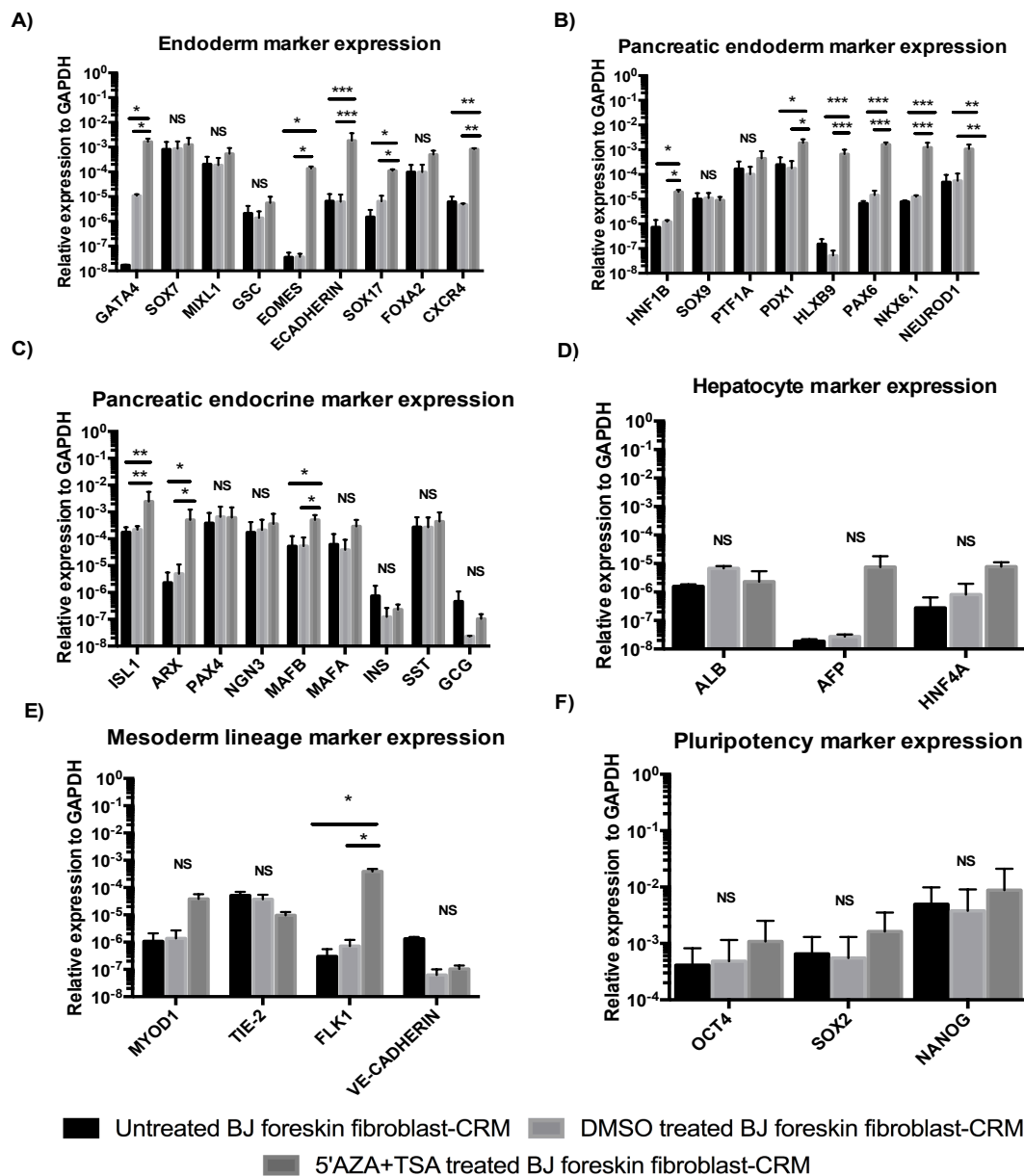


Figure-4: 5-azacytidine (5AZA) and Trichostatin-A (TSA) treatment of BJ foreskin fibroblast induces transient definitive endoderm and pancreatic endoderm markers. A) qRT-PCR analysis demonstrated induction of endodermal genes (*GATA4*, *EOMES*, *ECADHERIN*, *SOX17*, *CXCR4*, but not *SOX7*, *MIXL1*, *GSC*, *FOXA2*) in 5AZA+TSA CRM treated BJ foreskin fibroblast. B) qRT-PCR analysis demonstrated induction of pancreatic endoderm genes (*HNF1B*, *PDX1*, *HLXB9*, *PAX6*, *NKX6.1*, *NEUROD1*, but not *SOX9*, *PTF1A*) in 5AZA+TSA CRM treated BJ foreskin fibroblast. C) qRT-PCR analysis demonstrated induction of pancreatic endocrine genes (*ISL1*, *ARX*, *MAFB*, but not *PAX4*, *NGN3*, *MAFA*, *INS*, *SST*, *GCG*) in 5AZA+TSA CRM treated BJ foreskin fibroblast. D) qRT-PCR analysis demonstrated no induction of hepatocyte marker genes (*ALB*, *AFP*, *HNF4A*) in 5AZA+TSA CRM treated BJ foreskin fibroblast cells. E) qRT-PCR analysis demonstrated induction of mesoderm lineage genes (*FLK1* but not *MYOD1*, *TIE-2* and *VE-CADHERIN*) in 5AZA+ TSA CRM treated BJ foreskin fibroblast cells. F) qRT-PCR analysis demonstrated no induction of pluripotency genes (*OCT4*, *SOX2*, *NANOG*) in 5AZA+ TSA CRM treated BJ foreskin fibroblast cells. Black bar – CRM treated BJ foreskin fibroblast cells; Light grey bar-DMSO-CRM treated BJ foreskin fibroblast cells; Dark grey bar- 3 μ M-5AZA-CRM followed by 100 μ M TSA-CRM treated BJ foreskin fibroblast cells. Gene expression is shown relative to the housekeeping gene *GAPDH*. Data represent the mean \pm SEM (standard error of mean) of three independent experiments. Statistical significance tests were performed in between 5AZA+TSA treated versus untreated and 5AZA+TSA treated versus DMSO treated. * p <0.05, ** p <0.01 and *** p <0.001 by unpaired 2-tailed Student's t-test. NS- not significant

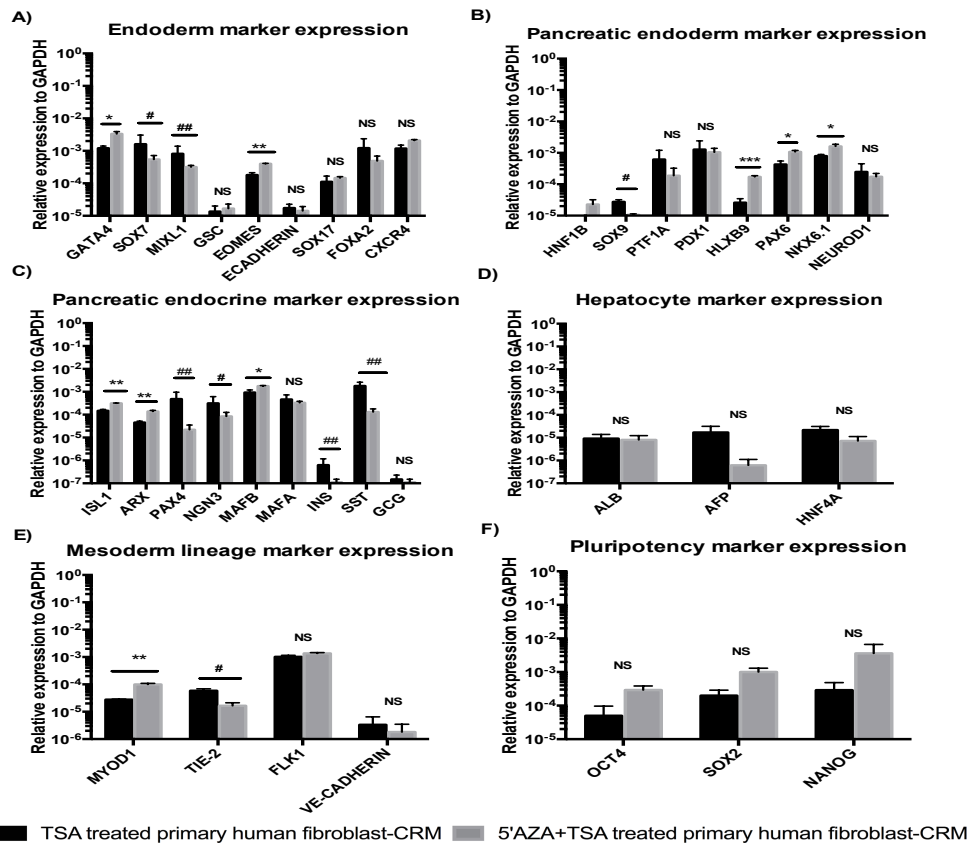


Figure-5: Comparison of gene expression changes in primary adult fibroblasts treated with TSA-CRM vs. 5AZA-TSA-CRM. A) Comparative qRT-PCR analysis demonstrated induction of endodermal genes (*GATA4*, *EOMES*)(* significant indicates) were more highly expressed in 5AZA+TSA CRM treated primary human fibroblast cells. However (*SOX7* and *MIXL1*) (# significant indicates) were induced more in TSA-CRM treated primary human fibroblast. No significant differences in (*GSC*, *E-CADHERIN*, *SOX17*, *FOXA2* and *CXCR4*) expression were found in 5AZA+ TSA CRM treated primary human fibroblast. B) Comparative qRT-PCR analysis demonstrated a greater induction of pancreatic endoderm genes (*HLXB9*, *PAX6*, *NKX6.1*)(* significant indicates) in 5AZA+ TSA CRM treated primary human fibroblast cells. However *SOX9* (# significant indicates) was induced more in TSA-CRM treated primary human fibroblast. No significant differences in (*HNF1B*, *PTF1A*, *PDX1* and *NEUROD1*) expression were found in 5AZA+ TSA CRM treated primary human fibroblast. C) Comparative qRT-PCR analysis demonstrated a greater induction of the pancreatic endocrine genes (*ISL1*, *ARX*, *MAFB*)(* significant indicates) in 5AZA+TSA CRM treated primary human fibroblast cells. However (*PAX4*, *NGN3*, *INS* and *SST*) (# significant indicates) were induced more in TSA-CRM treated primary human fibroblast. No significant differences in (*MAFB*, *GCG*) expression were found in 5AZA+ TSA CRM treated primary human fibroblast. D) Comparative qRT-PCR analysis demonstrated no differences in induction of hepatocyte genes (*ALB*, *AFP*, *HNF4A*) in 5AZA+TSA CRM treated primary human fibroblast. E) Comparative qRT-PCR analysis demonstrated a greater induction of the mesoderm lineage genes *MYOD1* (*Significant indicates) in 5AZA+ TSA CRM treated primary human fibroblast cells. However *TIE-2* (# significant indicates) was induced more in TSA-CRM treated primary human fibroblast. No significant differences in (*FLK1* and *VE-CADHERIN*) levels were found in 5AZA+ TSA CRM treated primary human fibroblast. F) Comparative qRT-PCR analysis demonstrated that no differences in induction of pluripotency genes (*OCT4*, *SOX2*, and *NANOG*) in 5AZA+ TSA CRM treated primary human fibroblast. Black bar – TSA-CRM treated primary human fibroblast cells. CRM; Dark grey bar-5AZA-CRM followed by 100μM TSA-CRM treated primary human fibroblast cells. Gene expression is shown relative to the housekeeping gene *GAPDH*. Data represent the mean ± SEM (standard error of mean) of three independent experiments. Statistical significance tests were performed in between 5AZA and followed by TSA treated versus TSA treated fibroblasts. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ and TSA treated versus 5AZA and followed by TSA treated fibroblasts. # $p < 0.05$, ## $p < 0.01$ by unpaired 2-tailed Student's t-test. NS- not significant.

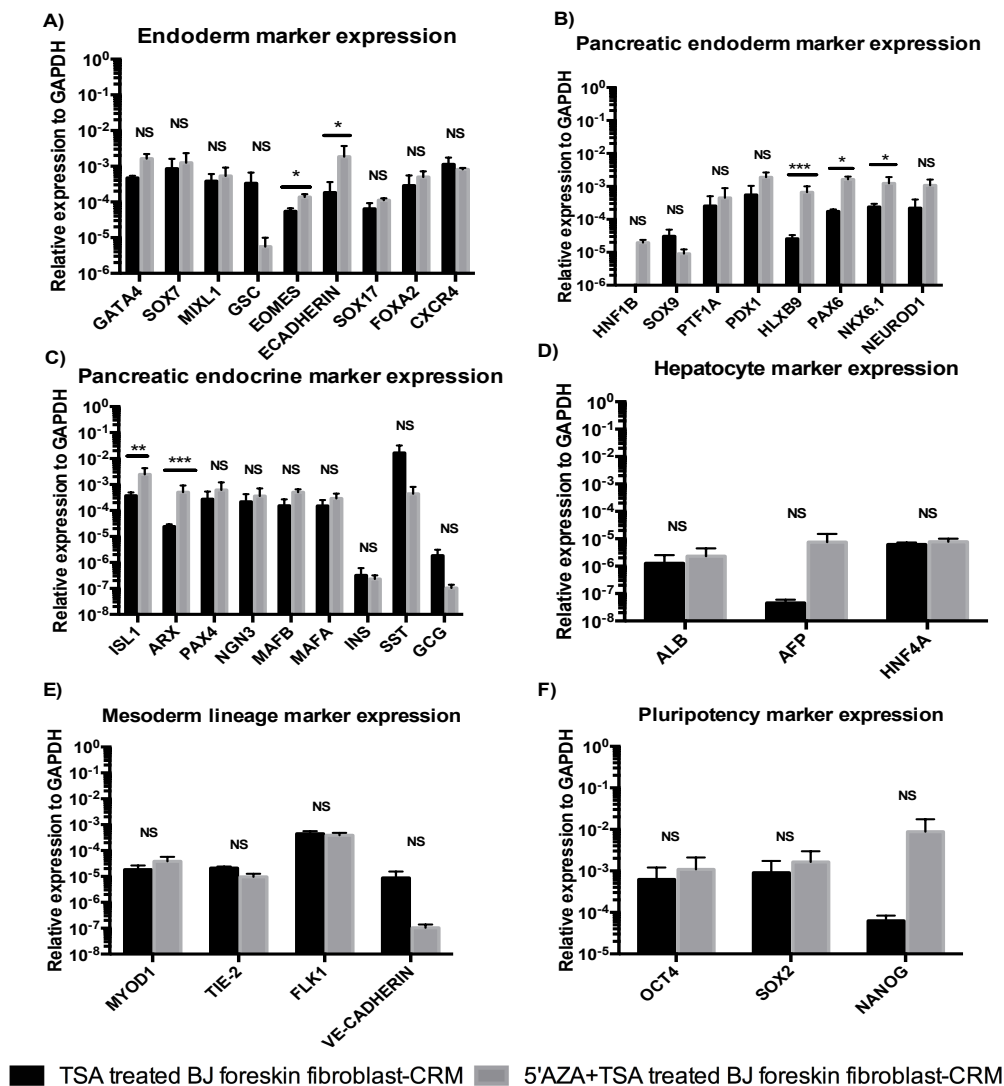


Figure-6: Comparison of gene expression changes in BJ1 foreskin fibroblasts treated with TSA-CRM vs. 5AZA-TSA-CRM. A) Comparative qRT-PCR analysis demonstrated induction of endodermal genes for (*EOMES*, *E-CADHERIN*) (* significant indicates) were more highly expressed in 5AZA+ TSA CRM treated BJ foreskin fibroblast. No significant differences in (*GATA4*, *SOX7*, *MIXL1*, *GSC*, *SOX17*, *FOXA2* and *CXCR4*) expression were found in 5AZA+TSA CRM treated BJ foreskin fibroblast. B) Comparative qRT-PCR analysis demonstrated significantly greater induction of pancreatic endoderm genes, (*HLXB9*, *PAX6*, *NKX6.1*) (* significant indicates) in 5AZA+ TSA CRM treated BJ foreskin fibroblast. No significant differences in (*HNF1B*, *SOX9*, *PTF1A*, *PDX1* and *NEUROD1*) expression were found in 5AZA+ TSA CRM treated BJ foreskin fibroblast. C) Comparative qRT-PCR analysis demonstrated greater induction of pancreatic endocrine genes (*ISL1*, *ARX*) (* significant indicates) in 5AZA+ TSA CRM treated BJ foreskin human fibroblast. No significant differences in (*PAX4*, *NGN3*, *MAFB*, *MAFA*, *INS*, *SST* and *GCG*) expression were found in 5AZA+ TSA CRM treated BJ foreskin human fibroblast. D) Comparative qRT-PCR analysis demonstrated no differences in induction of hepatocyte genes (*ALB*, *AFP*, and *HNF4A*) in 5AZA+ TSA CRM treated BJ foreskin human fibroblast. E) Comparative qRT-PCR analysis demonstrated the no differences in induction of mesodermal lineage genes (*MYOD1*, *TIE-2*, *FLK1*, and *VE-CADHERIN*) in 5AZA+ TSA CRM treated BJ foreskin human fibroblast. F) Comparative qRT-PCR analysis demonstrated no differences in induction of pluripotency genes (*OCT4*, *SOX2*, *NANOG*) in 5AZA+ TSA CRM treated BJ foreskin human fibroblast. Black bar – TSA-CRM treated BJ foreskin fibroblast cells; Dark grey bar- 5AZA-CRM followed by 100μM TSA-CRM treated BJ foreskin fibroblast cells. Gene expression is shown relative to the housekeeping gene *GAPDH*. Data represent the mean ± SEM (standard error of mean) of three independent experiments. Statistical significance tests were performed in between 5AZA and followed by TSA versus TSA treated fibroblasts. **p*<0.05, ***p*<0.01 and ****p*<0.001 by unpaired 2-tailed Student's t-test. NS- not significant.

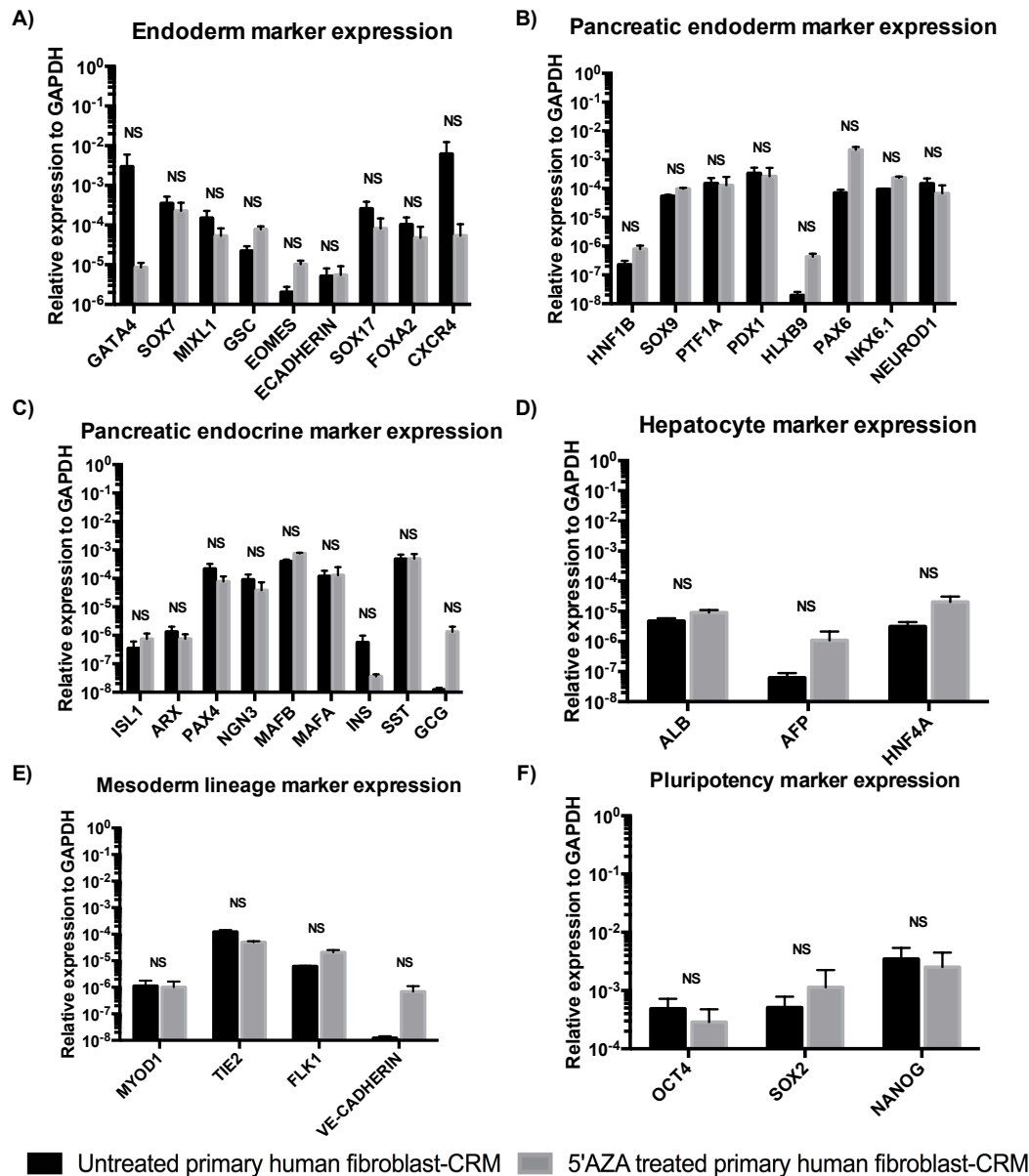


Figure-7: 5-azacytidine (5AZA) treatment of primary adult fibroblasts does not induce marker expression. A) qRT-PCR analysis demonstrated no differences in induction of endodermal genes for (*GATA4*, *SOX7*, *MIXL1*, *GSC*, *EOMES*, *E-CADHERIN*, *SOX17*, *FOXA2*, and *CXCR4*) in 5'AZA CRM treated primary human fibroblast. B) qRT-PCR analysis demonstrated no differences in induction of pancreatic endoderm genes (*HNF1B*, *SOX9*, *PTF1A*, *PDX1*, *HLXB9*, *PAX6*, *NKX6.1*, and *NEUROD1*) in 5'AZA CRM treated primary human fibroblast. C) qRT-PCR analysis demonstrated no differences in induction of pancreatic endocrine genes (*ISL1*, *ARX*, *PAX4*, *NGN3*, *MAFB*, *MAFA*, *INS*, *SST*, and *GCG*) in 5'AZA CRM treated primary human fibroblast. D) qRT-PCR analysis demonstrated no differences in induction of hepatocyte genes (*ALB*, *AFP*, *HNF4A*) in 5'AZA CRM treated primary human fibroblast. E) qRT-PCR analysis demonstrated no differences in induction of mesoderm lineage genes (*MYOD1*, *TIE2*, *FLK1*, and *VE-CADHERIN*) in 5'AZA CRM treated primary human fibroblast. F) qRT-PCR analysis demonstrated no differences in induction of pluripotency genes (*OCT4*, *SOX2*, *NANOG*) expression in 5'AZA CRM treated primary human fibroblast. Black bar – CRM treated primary human fibroblast cells; Dark grey bar–3 μ M 5AZA–CRM treated primary human fibroblast cells. Gene expression is shown relative to the housekeeping gene *GAPDH*. Data represent the mean \pm SEM (standard error of mean) of three independent experiments. Statistical significance tests were performed in between 5AZA-CRM treated primary human fibroblast versus untreated primary human fibroblasts in CRM by unpaired 2-tailed Student's t-test. NS- not significant.

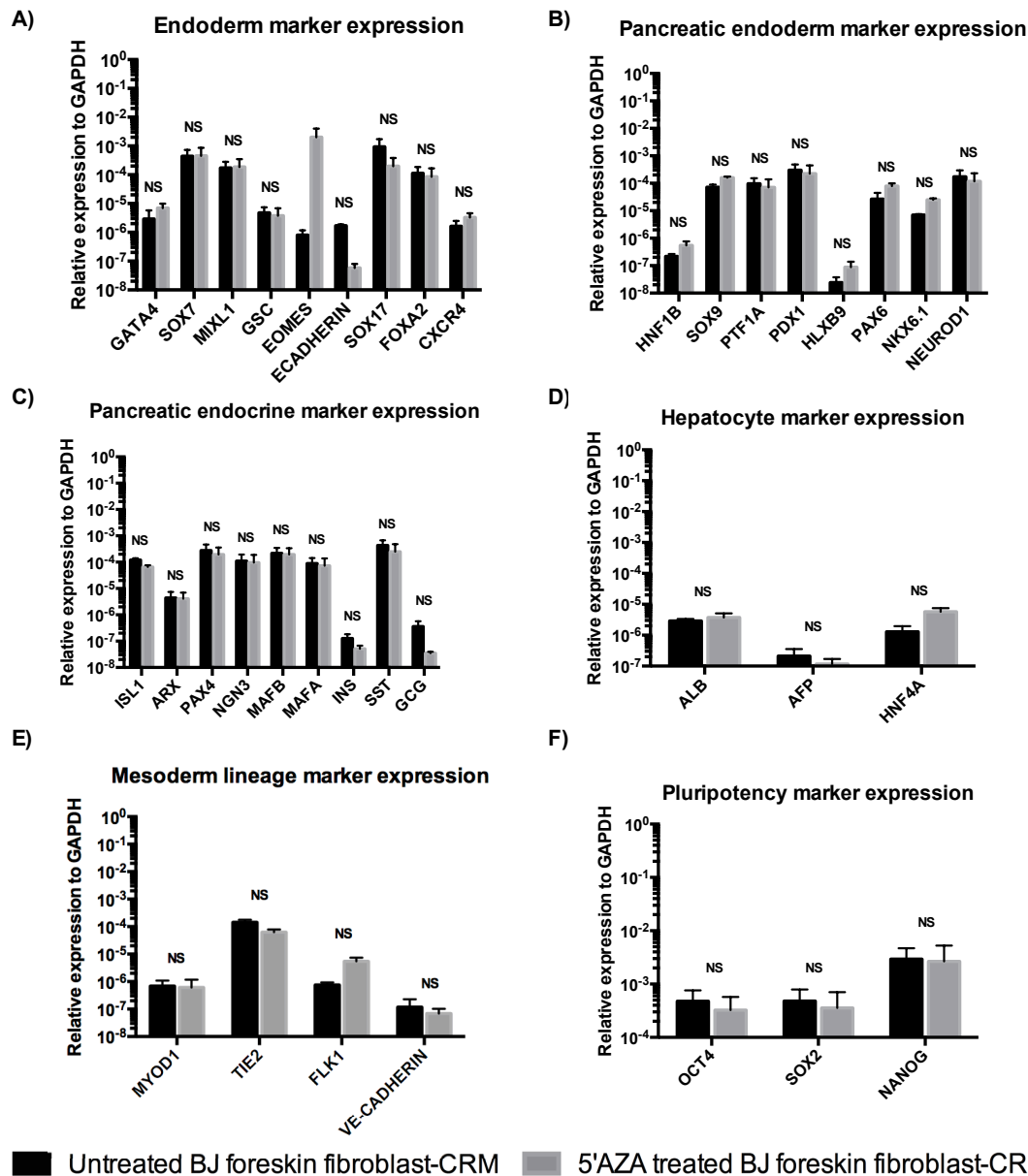


Figure-8: 5-azacytidine (5AZA) treatment of BJ foreskin adult fibroblast does not induces marker expression. A) qRT-PCR analysis demonstrated no differences in induction of endodermal genes for (*GATA4*, *SOX7*, *MIXL1*, *GSC*, *EOMES*, *E-CADHERIN*, *SOX17*, *FOXA2*, and *CXCR4*) in 5'AZA CRM treated BJ foreskin fibroblast. B) qRT-PCR analysis demonstrated no differences in induction of pancreatic endoderm genes (*HNF1B*, *SOX9*, *PTF1A*, *PDX1*, *HLXB9*, *PAX6*, *NKX6.1*, and *NEUROD1*) in 5'AZA CRM treated BJ foreskin fibroblast. C) qRT-PCR analysis demonstrated no differences in induction of pancreatic endocrine genes (*ISL1*, *ARX*, *PAX4*, *NGN3*, *MAFB*, *MAFA*, *INS*, *SST*, and *GCG*) in 5'AZA CRM treated BJ foreskin fibroblast. D) qRT-PCR analysis demonstrated no differences in induction of hepatocyte genes (*ALB*, *AFP*, *HNF4A*) in 5'AZA CRM treated BJ foreskin fibroblast. E) qRT-PCR analysis demonstrated no differences in induction of mesoderm lineage genes (*MYOD1*, *TIE2*, *FLK1*, and *VE-CADHERIN*) in 5'AZA CRM treated BJ foreskin fibroblast. F) qRT-PCR analysis demonstrated no induction of pluripotency genes (*OCT4*, *SOX2*, *NANOG*) expression in 5'AZA CRM treated BJ foreskin fibroblast. Black bar – CRM treated BJ foreskin fibroblast cells; Dark grey bar-3 μ M 5AZA-CRM treated BJ foreskin fibroblast cells. Gene expression is shown relative to the housekeeping gene *GAPDH*. Data represent the mean \pm SEM (standard error of mean) of three independent experiments. Statistical significance tests were performed in between 5AZA-CRM treated BJ foreskin fibroblast versus untreated BJ foreskin fibroblasts in CRM by unpaired 2-tailed Student's t-test. NS- not significant.

5.5 Discussion

We demonstrated that treatment of two different sources of human fibroblasts with TSA in CRM induced expression of endoderm lineage markers and pancreatic endoderm markers known to be important for β -cell differentiation, albeit at modest levels. By contrast, 5AZA did not induce expression of endoderm and pancreatic endoderm markers though morphological changes occurred. However, the induction resulting from treatment with TSA, or a combination of 5AZA followed by TSA was reversible and disappeared upon removal of the epigenetic modifiers and the chromatin remodeling medium. Interestingly, already in 1977, Taylor and Johns demonstrated that 5AZA can support transdifferentiation of mouse fibroblasts into skeletal myoblasts by the master regulator *MyoD1* [29, 30] but did not recognize the involvement of epigenetics.

In line with our studies, Pennarossa et al., [19] described that culture of human fibroblasts with 5AZA resulted in minimal induction of endodermal and pancreatic marker genes. However, they demonstrated that such pretreatment allows cells subsequently to differentiate into β -cells when cultured in the presence of cocktails of growth factors to induce pancreatic endoderm and subsequently mature endocrine pancreatic cells. In a second study by the same group [20], similar results were reported for pig fibroblasts, where again they described that treatment with 5AZA alone does not cause robust changes in endoderm and pancreatic gene expression patterns, but may change the epigenetic state of fibroblasts such that they subsequently become susceptible to growth factors that support pancreatic endocrine and β -cell differentiation.

Another approach, described by Katz et al., combined the use of TF overexpression followed by treatment of the transduced cells on day 3 with a HDACi (Romidepsin) and 5AZA to transdifferentiate human dermal fibroblasts into β -cells [21]. They demonstrated that Romidepsin could activate some endocrine pancreas genes, whereas 5AZA alone did not. This corroborates our observations that the HDAC inhibitor TSA can induce endoderm and pancreatic marker genes. They further found

that *PDX1* was only minimally induced despite the fact that the combination of Romidepsin and 5AZA could induce a number of markers of pancreatic endoderm and more mature pancreatic cells. Only when overexpression of *PDX1* was combined with the epigenetic modifiers human fibroblasts could be reprogrammed to β -cells.

We speculate that TSA combined with CRM may induce a “versatile” epigenome surrounding endodermal and pancreatic genes, but cannot induce full reprogramming. Full reprogramming may require that we combine this approach with transduction of a limited number of key pancreatic TFs (like *PDX1*) or culture in growth factor cocktails known to allow differentiation of PSC to β -cells. Aside from the HDACi, TSA, CRM was also needed to induce the endodermal and pancreatic genes. Which factor from CRM has this added epigenetic effect is currently still unclear, but it might be L-Ascorbic acid, as this compound has been shown to enhance reprogramming via the induction of Ten-eleven translocation (TET) hydroxylase dependent DNA demethylation [31-33].

5.6 Conclusion

In conclusion, we described an optimized schedule for treatment of human fibroblasts with epigenetic modifiers to activate endocrine pancreatic marker genes. We demonstrated that culture of adult human fibroblasts with and TSA combined with CR medium, induces expression of endoderm and pancreatic endoderm genes, but that this is a reversible phenomenon. Treatment with TSA/CRM also induced some mesoderm lineage markers *MYOD1* and *FLK1* in both fibroblast cell lines. When treatment with 5AZA-CRM was added before TSA-CRM treatment, an increase in mesoderm lineage markers *MYOD1* was seen beyond levels induced by TSA-CRM, but 5AZA/CRM itself did not induce any lineage marker expression. Hence, further maintenance in β -cell-specifying conditions with or without forced expression of exogenous TFs will be needed to eventually convert fibroblasts to β -cells.

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Supplementary table-1

Primers sets used for gene expression analysis by qRT-PCR

Gene	Forward	Reverse
<i>GAPDH</i>	TCAAGAAGGTGGTGAAGCAGG	ACCAGGAAATGAGCTTGACAAA
<i>GATA4</i>	TCCAAACCAGAAAACGGAAG	CTGTGCCCCGTAGTGAGATGA
<i>SOX7</i>	GCCTGTGCAACAAGAGTGAA	GTACCCTGGGTCTTTGGTCA
<i>MIXL1</i>	GGATCCAGGTATGGTTCCAG	CATGAGTCCAGCTTTGAACC
<i>GSC</i>	TCTCAACCAGCTGCACTGTC	CCAGACCTCCACTTTCTCCTC
<i>EOMES</i>	AACAACACCCAGATGATAGTC	TCATAGTTGTCTCTGAAGCCT
<i>ECADHERIN</i>	CGAACTATATTCTTCTGTGAGAGG	GATAGATTCTTGGGTGGGTC
<i>SOX17</i>	CGCTTTCATGGTGTGGGCTAAGGACG	TAGTTGGGGTGGTCTGCATGTGCTG
<i>FOXA2</i>	AGGAGGAAAACGGGAAAGAA	GGTGCTTGAAGAAGCAGGAG
<i>CXCR4</i>	CACCGCATCTGGAGAACCA	GCCCATTTCTCGGTGTAGTT
<i>HNF1B</i>	TCACAGATACCAGCAGCATCAGT	GGGCATCCCAGGCTTGTA
<i>SOX9</i>	ACGCCATCTTCAAGGCGCTG	CCGGCTGCACGTCGGTTTT
<i>PTF1A</i>	ACGACTTCTTCAACGACCAG	TGGTGGCTAAGGAACTCCAC
<i>PDX1</i>	TCCACCTTGGGACCTGTTTA	GTGTGTTAGGGAGCCTTCCA
<i>HLXB9</i>	ATGATCCTGCCTAAGATGCC	AAATCTTCACTGGGTCTCG
<i>PAX6</i>	CCCAAGAGCAAATTGAGGCC	CTCTTCTCCATTTGGCCCTTCGA
<i>NKX6.1</i>	CTTCCCGTCTTTGTCCAACAA	CCATCTTCTGGCCCGAGTGA
<i>NEUROD1</i>	TAAGACGCAGAAGCTGTCCA	CTGCTCAGGCAGAAAAGTCC
<i>ISL1</i>	GTACAACCACCATTTCACTG	CCCGTACAACCTGATATAATCTC
<i>ARX</i>	ACAGCGTGTGCCTCTCTGC	TCGGGCCTCGGTCAAGTCC
<i>PAX4</i>	CAACCGAGTCCTGCGGGCAT	GCCAGCTTCCACGGGCCAC
<i>NGN3</i>	TCTCTATTCTTTGCGCCGG	CTTGGACAGTGGGCGCAC
<i>MAFB</i>	GCCTGCGCTAATTGTAGGAG	CAAAAGCAGGGAAAGAAACG
<i>MAFA</i>	TCATCCGGCTCAAGCAGAAG	TCTCGCTCTCCAGAAATGTGC
<i>INS</i>	ATCAAGCACATCACTGTCCT	TGTAGAAGAAGCCTCGTTCC
<i>SST</i>	GAGGCTTGAGCTGCAGAGAT	TCGCTGAAGACTTGGAGGAT
<i>GCG</i>	GTTCCCTTCAAGACACAGAG	GGCAATGTTATTCTGTTC
<i>MYOD1</i>	CGACGGCATGATGGACTACA	TAGTAGGCGCCTTCGTAGCA
<i>TIE2</i>	TGCCCAGATATTGGTGTCT	CTCATAAAGCGTGGTATTCACGTA
<i>FLK1</i>	ACAACCAGACGGACAGTGGT	AGCCTTCAGATGCCACAGAC
<i>VE-CADHERIN</i>	GTTACGCATCGTTGTTC	TCTGCATCCACTGCTGTCA
<i>ALB</i>	ATGCTGAGGCAAAGGATGTC	AGCAGCAGCACGACAGAGTA
<i>AFP</i>	TGAGCACTGTTGCAGAGGAG	GTGGTCAGTTGCAGCATTC
<i>HNF4A</i>	ACTACGGTGCCTCGAGCTGT	GGCACTGGTTCCTCTTGTCT
<i>OCT4</i>	GATGGCGTACTGTGGGCC	TGGGACTCCTCCGGGTTTTG
<i>SOX2</i>	TGGCGAACCATCTCTGTGGT	CCAACGGTGTCAACCTGCAT
<i>NANOG</i>	CCTGTGATTGTGGGCCTG	GACAGTCTCCGTGTGAGGCAT